

**AMPLIFICATION OF HERPES SIMPLEX VIRUS TYPES 1 AND 2
AND HUMAN HERPES VIRUS TYPE 5 POLYMERASE GENE
SEGMENT FROM FORMALIN-FIXED BRAIN TISSUE
FROM ALZHEIMER'S DISEASE PATIENTS**

by

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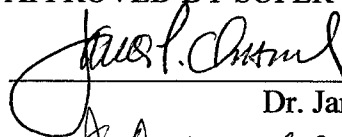
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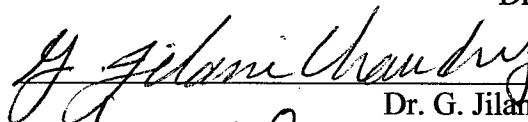
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It is known that nucleic acids from formalin-fixed tissues are not nearly as good templates for DNA amplification as those extracted from fresh tissue. However, specimens stored in most pathologic archives are initially fixed in formalin. The possibility of an infectious etiology of several diseases, including Alzheimer's disease (AD), underscores the usefulness of archived tissue in assessing the association of infectious agents with specific pathology. Previous studies have used frozen brain tissue samples and cerebrospinal fluid (CSF) specimens to determine whether Herpes simplex virus type 1 (HSV1) is present more frequently in Alzheimer's disease brain tissue than in the brains of elderly normal subjects. The DNA extraction method used in this study resulted in extraction of DNA from formalin-fixed brain tissue samples. The neuronal nitric oxide synthase (NOS1) gene target was amplified and sequenced in all samples tested, in addition to HSV1, HSV2, or Human Herpes virus type 5 (HHV5) from respective samples.

TABLE OF CONTENTS

Acknowledgements	iii
Abstract	iv
List of Figures	vi
List of Tables	vi
List of Abbreviations	vii
Introduction	1
Materials	5
Methods	6
A. Formalin Removal	6
B. DNA Extraction	7
C. PCR	8
D. Reamplification of PCR Product	9
E. Purification of Amplified Product	10
F. Sequencing	10
Results	12
Discussion	16
References	18
Vita	

LIST OF FIGURES

Fig 1: Agarose Gel Electrophoresis of NOS1 Gene Target.....	13
Fig 2: Agarose Gel Electrophoresis of Herpes Virus Polymerase Gene Targets.....	13
Fig 3: NOS1 Gene Target Sequence.....	14
Fig 4: Sequence of HSV1, HSV2, and HHV5 Polymerase Gene Targets.....	14

LIST OF TABLES

Table 1: Herpes Virus Positive Sequence Results.....	15
Table 2: Percent Samples Testing Positive for Herpes Virus by Type.....	15

LIST OF ABBREVIATIONS

AD	Alzheimer's disease
ApoE	Apolipoprotein E
bp	Base Pair
CSF	Cerebrospinal Fluid
CMV	Cytomegalovirus
ddNTP	Dideoxy Nucleotide Triphosphate
DNA	Deoxyribonucleic acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HSV	Herpes Simplex Virus
HSV1	Herpes Simplex Virus Type 1
HSV2	Herpes Simplex Virus Type 2
HHV5	Human Herpes Virus Type 5
HHV6	Human Herpes Virus Type 6
NOS1	Neuronal Nitric Oxide Synthase
PBS	Phosphate Buffered Saline
PE	Phosphate Ethanol
PCR	Polymerase Chain Reaction

INTRODUCTION

Alzheimer's disease (AD) is an age-related, irreversible neurodegenerative disorder. AD results in memory loss and a decline in cognitive function which ultimately leads to a total degradation of intellectual and mental capabilities. AD is the leading cause of dementia with 20 million reported cases worldwide. Afflicting 1 in 5 people over the age of 80, AD is responsible for the deaths of one half million Americans each year (1). Although not the cause of AD, current studies suggest that HSV1 infection of the brain is a significant risk factor. HSV1 infection potentially predisposes individuals to increased inflammation, neuritic plaque and neurofibrillary tangle formation, and beta-amyloid deposition.

The Herpes simplex viruses (HSV) belong to the alpha herpesvirus subfamily of herpesviruses. These enveloped viruses possess a double stranded DNA genome approximately 150 kb in size. The genome of Herpes simplex virus type 1 (HSV1) and Herpes simplex virus type 2 (HSV2) share 50-70 % homology. HSV1 is primarily associated with oral and ocular lesions, while HSV2 is primarily associated with genital and anal lesions. During the primary infection stage, the virus enters peripheral sensory nerves and migrates along axons to sensory nerve ganglia in the CNS allowing the virus to escape the immune surveillance system. Reactivation of latent virus in nerve cells leads to recurrent disease. The virus travels back down sensory axons where it establishes an infection in the epithelium of the skin and replicates.

Human Herpes virus type 5 (HHV5), also known as Cytomegalovirus (CMV), belongs to the beta herpesvirus subfamily of herpesviruses and like the alpha subfamily members is enveloped, possessing a slightly larger double stranded DNA genome of approximately 230 kb. HHV5 infection is common and most infections are asymptomatic. Apart from during pregnancy and newborn infants exposed *in utero*, active HHV5 infection only occurs in individuals with

immune defects such as AIDS and immunosuppressed organ transplant patients. Transmission is believed to be by oral/respiratory route.

HSV1 has been proposed as a possible factor in Alzheimer's disease based on the following: several neurological disorders are caused by common viruses; HSV1 is ubiquitous with predilection for latent residence in the peripheral nervous system; and in acute HSV1 encephalitis, the virus targets the same regions of the central nervous system (temporal and frontal cortex, and hippocampus) as those most affected in AD (2).

It has been established using the polymerase chain reaction (PCR) that HSV1 is present in a high proportion of brains of elderly normal subjects and AD patients (3). In younger individuals, the virus is almost always absent in brain. It was therefore suggested that HSV1 reaches the brain in older age as the immune system declines and resides there in latent form. Under conditions of stress or immunosuppression, HSV1 reactivates and gives rise to an acute but localized infection, the damage being more serious in people who possess an APOE-ε4 allele (4).

Under appropriate circumstances, the other human herpesviruses can all be neuroinvasive and could therefore act as another possible risk factor for AD. Although rare, HSV2 can also give rise to brain stem encephalitis and has been shown to be the cause of a progressive dementia syndrome diagnosed as AD based on clinical criteria (3). The nervous system is one of the main targets in CMV congenital infection. Mental retardation is common-place in survivors of congenital CMV infection. The virus can also cause encephalitis; although, it is rare in immunocompetent people (3).

Neuronal nitric oxide synthase (NOS1) gene is a universal mediator of biological effects in the brain. NOS1 is involved in many biological systems; neurotransmission, the regulation of body fluid homeostasis, neuroendocrine physiology, control of some muscle, motility, sexual

function, and myocyte/myoblast biology (5). In all experiments performed using formalin-fixed brain tissue described in this study, the NOS1 gene was consistently amplified. Thus, NOS1 proved to be a better internal control standard than that of the housekeeping gene, glyceralde-3-phosphate dehydrogenase (GAPDH). Thus, the NOS1 gene was used as an internal control to verify successful removal of formalin that inhibits PCR amplification of DNA. The NOS1 gene is located on human chromosome 12 and spans nearly 150 kb. The gene is comprised of 29 exons and 28 introns. The nucleotide sequence located on exon 19 was used in assigning forward and reverse primers giving rise to 251 bp amplicon.

The focus of this thesis is examination of formalin-fixed, post-mortem brain tissue from patients with Alzheimer's disease for the presence of HSV1, HSV2, and HHV5 DNA using PCR. A number of studies have been completed over the past 20 years to assess whether the presence of HSV1 in aged brain is associated with AD using frozen post-mortem brain tissue sections and cerebrospinal fluid (CSF) specimens. However, little research has been conducted determining the presence of viral DNA in formalin-fixed brain tissue.

Although 10 % formalin (3.7 % formaldehyde) treatment of tissue is associated with deleterious interactions of varying degree with DNA (6-11), formalin provides the most adequate means of tissue preservation insuring optimal features of histological purposes (11).

In 1990, Nicoll, et al. detected viral DNA using formalin-fixed, paraffin-embedded brain samples from patients with acute HSV1 encephalitis by PCR (13). Bertrand and coworkers subsequently described PCR based detection of HSV1 viral DNA in specific formalin-fixed brain tissue regions of Alzheimer patients (14). Recently, Hemling and coworkers detected HSV1, Human Herpes virus type 6 (HHV6) and varicella zoster viral DNA using PCR in human brain, but the source of PCR template DNA is unclear (14). However, using the methods of Nicoll

(13), Bertrand (14), and Hemling (15), we were not successful in detecting by PCR the key glycolytic enzyme (GAPDH) commonly used as a marker to exclude false negative results. Assay variation could arise from differences in inherent molecular profiles; however, such intrinsic tissue factors affecting PCR amplification of template DNA in archived tissue are not easily identified.

In this study, the presence of HSV1, HSV2 and HHV5 was detected in formalin-fixed brain tissue sections from patients with AD using PCR. This study demonstrated the utility of post-analysis nucleic acid extraction developed in this laboratory and PCR amplification using formalin-fixed tissue. Considering the large number of archived tissue samples available, the procedure employed in this study could be used in assessing the association of infectious agents with specific pathology in archived brain tissue.

MATERIALS

Proteinase K, Cell Lysis Solution and MPC Protein Precipitation Reagent were obtained from Epicentre Biotechnologies, Madison, Wisconsin. Xylene, 100 % Ethanol and 99 % (v/v) Isopropanol were obtained from Sigma-Aldrich Co., St. Louis, Missouri. Nuclease Free double distilled water was obtained from Ambion Inc., Austin, Texas. HSV1 and HSV2 viral cultures from Cell Lines A549 and MRC5 were obtained from Brooks City Base, San Antonio, Texas. HHV5 has obtained from American Type Culture Collection, Manassas, Virginia. The universal primer set specific for the polymerase HSV1, HSV2, and HHV5 gene sequences and primer set specific for the NOS1 gene target were obtained from Integrated DNA Technologies, Inc., Coralville, Iowa. Reaction Mix 2X from the 'Superscript II One Step-RT-PCR', Platinum Taq DNA Polymerase and 2 % (w/v) E-Gel^R Agarose gels were obtained from Invitrogen Corporation, Carlsbad, California. Molecular weight ladder (100 bp) were obtained from Bio-Rad Laboratories Inc., Hercules, California. Human Genomic DNA was obtained from Roche Diagnostics GmbH, Penzberg, Germany. Ultracentrifuge-MC centrifugal filter devices were obtained from Amicon Corporation, Billerica, Massachusetts. QIAquick PCR Purification Kit and DyeExTM 2.0 Spin Kit were obtained from Qiagen Inc., Valencia, California. Big Dye Terminator v3.1 Cycle Sequencing RR-100 and Formamide were obtained from Applied Biosystems, Foster City, California. Feather Sterile Surgical Blade and Fisherbrand 2 mL and 1.5 mL microcentrifuge tubes were obtained from Fisher Scientific International Inc., Houston, Texas. Formalin-fixed Alzheimer patient brain tissue was obtained from the Honolulu Heart Program, Honolulu, Hawaii.

METHODS

The extraction and amplification of DNA from formalin-fixed brain tissue used in this study is a multi-step process and is summarized below.

Step 1: Tissue preparation

Step 2: Xylene extraction (formalin removal)

Step 3: Cell lysis/protein digestion

Step 4: PCR amplification

Step 5: Agarose gel electrophoresis of amplicons

Step 6: Amplicon cleanup

Step 7: Sequencing

A. Formalin Removal

Formalin-fixation reduces significantly the success of PCR amplification primarily because of modification and cross-linking of DNA. Although not firmly established, it appears that xylene treatment in addition to removing formalin also removes residual inhibitors derived from formalin-fixation. Approximately 10-15 mg of tissue was processed by mincing the tissue using a sterile surgical blade. Once completed, the finely minced tissue was transferred to a 2 mL microcentrifuge tube and to each sample 1 mL xylene was added. The contents were then vortexed vigorously for 5 seconds, followed by incubation at room temperature for 15 minutes. Xylene was decanted and discarded using a pipette. This step was repeated twice. Residual xylene removal was accomplished by addition of 1 mL ACS grade, 100 % ethanol, vortexed for 5 seconds, and incubated at room temperature for 5 minutes. This procedure was repeated once. Samples were desiccated for at least 30 minutes in order to thoroughly dry the pellets removing residual xylene which inhibits subsequent Proteinase K digestion.

B. DNA Extraction

Successful DNA extraction requires that cells be lysed making the DNA assessable for subsequent steps. Proteinase K enzymatically degrades protein which aids lysis, freeing up DNA from associated protein. Nucleic acids are precipitated by addition of isopropyl alcohol to the supernatant material following centrifugation of the Proteinase K digest mixture. DNA extraction was accomplished using modification of the Epicentre MasterPure reagent DNA extraction procedure (16). Tissue and Cell Lysis Solution (300 ul) containing 2 ul 50 mg/ml Proteinase K and 10 ul 50 ug/ul Proteinase K in a total volume of 312 ul was added to each sample. The reaction mixture containing the tissue sample and Proteinase K cocktail was mixed thoroughly and incubated at 65° C overnight. Following incubation, samples were placed on ice for 4 minutes. To the lysed sample, 150 ul MPC Protein Precipitation Reagent was added, gently agitated for 10 seconds and resulting debris removed by centrifugation for 10 minutes at 9,000 x g using a microcentrifuge. Supernatant material was carefully decanted and transferred to a 2 mL microcentrifuge tube. Isopropyl alcohol (500 ul) (99 % (v/v) molecular biology grade) was added to the supernatant and each sample was inverted multiple times (~50). DNA was pelleted by centrifugation at 5° C for 10 minutes at 9,000 x g. Isopropyl alcohol was carefully aspirated without dislodging the DNA pellet. The pellet material was washed twice with 35 ul 75 % (v/v) molecular biology grade ethanol, gently tapping the tube for approximately 8 seconds each wash. Xylene (200 ul) was added to the respective DNA pellets, incubated at room temperature for 15 minutes followed by bulk removal of xylene using a pipette. Residual xylene was removed by adding to each sample 500 ul 100 % ethanol, followed by incubation at room temperature for 5 minutes. Samples were desiccated for 30 minutes in order to remove residual

ethanol followed by resuspension in 20 ul nuclease free water. This material served as DNA template for PCR amplification.

C. PCR

PCR amplifies the desired targeted DNA sequence giving rise to large amounts of amplified product (amplicons). Control DNA obtained from HSV1, HSV2, and HHV5 cultures and tissue extracted DNA was quantitated spectrophotometrically. Tenfold dilutions ranging from 100 ng/ul to 1 pg/ul were made for each of the templates using nuclease-free water. Detection of HSV1, HSV2 and HHV5 template control DNA was observed in a range of 100 ng/ul to 1 pg/ul under the conditions described below. To each reaction was added 2 ul of each DNA dilution per 20 ul total reaction volume. A universal primer set derived from the HSV1, HSV2 and HHV5 viral polymerase gene sequences produced expected PCR amplicon products of 235 (HSV1 and HSV2) and 238 (HHV5) base pairs using the forward and reverse primer sequences 5'-TCATCTACGGGGACACGGAC-3' and 5'-TTGCGCACCAGATCCACG-3', respectively. The nucleotide sequence from the NOS1 gene served as an internal control and was amplified (251 bp product). The NOS1 forward primer sequences used were: 5'-GGCCAACAATTCCC-TCATCAGCAA-3' and reverse 5'-TGAGTGTGGGAGAGCAGCAAAGTA- 3', respectively.

PCR reactions (20 ul total volume) for amplification of herpes viral and NOS1 gene targets were carried out using 2X Superscript II one Step-RT-PCR with Platinum Taq system reaction master mix at a final 1X concentration containing 0.5 nM of each primer and 0.1 U/ul Platinum Taq DNA polymerase. Reaction volumes were all brought to 20 ul with nuclease-free water. Conditions for amplification of herpes virus gene targets were as follows: initial denaturation for 3 minutes at 95° C, followed by 35 cycles consisting of DNA melting for 30 seconds at 95° C, primer-annealing for 15 seconds at 54° C, and primer elongation at 68° C for 30 seconds. The

final elongation reaction was carried out for 7 minutes at 68° C. Conditions for amplification of NOS1 gene was as follows: initial denaturation for 2 minutes at 94° C, followed by 35 cycles consisting of DNA melting for 15 seconds at 94° C, primer-annealing at 15 seconds at 52° C and primer elongation at 72° C for 20 seconds. The final elongation reaction was carried out for 5 minutes at 72° C. All PCR reactions were carried out using a Robocycler 96 Gradient Cycler with hot top (Stratagene, La Jolla, California).

PCR products were separated by electrophoresis using 2 % (w/v) E-Gel agarose gels containing ethidium bromide and visualized under Fisher Biotech UV Transilluminator (Fisher Scientific International Inc., Vernon Hills, Illinois). The respective amplicon products were size characterized using markers of known size. Stained gels were archived using a Fisher Biotech photo-documentation camera (Fisher Scientific International Inc., Vernon Hills, Illinois).

D. Reamplification of PCR Product

Reamplification of amplicon material was carried out after removing the respective gel areas containing the bands from the gel using a surgical blade. Each gel slice was transferred to an Ultracentrifuge-MC centrifugal filter. The centrifugal filter is designed to extract 100 to 10,000 bp DNA segments from agarose gel slices. The device utilizes gel compression to extract DNA from the agarose. Centrifugal force collapses the gel structure pushing the agarose through the gel nebulizer as a slurry that is captured by the Ultrafree-MC filter. Extruded DNA dissolved in electrophoresis buffer passes through the membrane and collects in the filtrate vial. The Ultracentrifuge-MC centrifugal filter device was centrifuged at 9,000 x g for 10 minutes using an Eppendorf Centrifuge 5417C (Eppendorf AG, Hamburg, Germany). Respective filters were discarded and eluent material used for reamplification. Reamplification was carried out using

2 ul gel slice eluent material under conditions identical to those described above. Re-amplification of the product was verified by agarose gel electrophoresis as previously described.

E. Purification of Amplified Product

Amplified products were cleaned up using the manufacturer's protocol for the QIAquick PCR Purification Kit. The protocol is designed to purify single or double stranded DNA fragments from PCR reactions. Amplicons ranging from 10 to 100 kb are purified away from primers, nucleotides, polymerases and salts using the QIAquick spin columns in a microcentrifuge. DNA i.e., amplicons, adsorb to the silica-membrane in the presence of high salt while contaminants pass through the column. After the impurities are washed away, the pure DNA is eluted from the silica-membrane with Tris buffer.

All centrifugation steps were carried out at 15,600 x g in an Eppendorf 5417C tabletop centrifuge. Five volumes of PBS were added to one volume of sample and mixed well. The sample was applied to a QIAquick spin column collection tube and centrifuged for 60 seconds. Flow-through was discarded. PE buffer (750 ul) was added to the column and centrifuged for 60 seconds. Flow-through material was discarded and bound DNA was eluted by adding 30 ul elution buffer (allowed to stand for 1 minute), followed by centrifugation for 60 seconds.

F. Sequencing

Sequencing was carried out to validate the amplicons produced following amplification. Sequencing is based on the same principle as that of the Sanger DNA method of sequencing. The ddNTP's are fluorescently labeled with different dyes and act as chain terminators. The four dyes fluoresce at different wavelengths. A laser determines the identity of each according to the wavelength at which it fluoresces. Results are expressed in the form of a chromatogram i.e., a

diagram of colored peaks corresponding specifically to the nucleotide in that location in the sequence.

Typical sequencing reactions consisted of 4 ul Big Dye Terminator v3.1 Cycle sequencing RR-100, 5 ul nuclease free water, and 1 ul 20 uM primer and 10 ul template. Cycle conditions were 95° C for 2 minutes, followed by 35 cycles of 96° C for 5 seconds, 50° C for 5 seconds and 60° C for 4 minutes.

Excess dye terminators were removed using a DyeExTM 2.0 Spin Kit per manufacturer's instructions. The DyeEx procedure is based on separation properties of gel-filtration chromatography. When sequencing reaction contents are applied onto DyeEx modules, dye terminators diffuse into the pores and are retained in the gel-filtration material while the DNA fragments are excluded and recovered in the flow-through. All centrifugation steps were carried out in an Eppendorf Centrifuge 5417C at 700 x g. The spin column was gently vortexed to re-suspend the resin. The cap of the tube was loosened a quarter turn. The bottom closure of the spin column was snapped off and the column placed in a 2 mL collection tube. The column was centrifuged for 3 minutes at the calculated speed and transferred to a 1.5 mL centrifuge tube. A 10 ul sequencing reaction aliquot was then applied to the gel bed. The column was centrifuged for 3 minutes at the calculated speed. Respective spin column eluates were dehydrated using a vacuum centrifuge followed by resuspension in 10 ul formamide and samples were subsequently sequenced using an ABI-3100 automated sequencer. (Applied Biosystems, Foster City, California).

RESULTS

Brain tissue from 57 elderly subjects clinically diagnosed with AD were investigated for HSV1, HSV2 and HHV5 polymerase gene targets using PCR. The integrity of the DNA samples used in this study were confirmed by demonstration of amplification of the NOS1 gene following DNA extraction from formalin-fixed tissue samples. A representative gel electrophoretic analysis of NOS1 gene (251 bp) PCR product, i.e., amplicons from formalin-fixed human brain samples positive for the presence of herpes viral DNA is shown in Figure 1. Human genomic DNA was used as a positive control. Reaction mixture without template served as the negative control (no-DNA).

A representative electrophoretic analysis of amplicons produced by amplification of HSV1 and HSV2 (235 bp) and HHV5 viral polymerase (238 bp) gene targets from formalin-fixed human brain is shown in Figure 2. Viral control DNA obtained from HSV1, HSV2, and HHV5 viral cultures was used for size comparison of amplicons.

Reamplification of respective gel slice extracted bands and subsequent sequence analysis confirmed the identity of the amplicons. All nucleotide sequences were subjected to a PubMed BLAST search and all sequences correlated to known HSV sequences shown in Figures 3 and 4. As shown in Tables 1 and 2, 11 of the 57 AD samples were found to be positive for herpes virus sequences (3 HSV1, 5 HSV2 and 3 HHV5).

Figure 1. Agarose Gel Electrophoresis of NOS1 Gene Target.

Electrophoretic analysis of NOS1 gene amplicon (251 bp) from formalin-fixed brain tissue samples was carried out as previously described. Lane 1, 100-bp molecular weight ladder. Lane 2, GAPDH control. Lanes 2-10, HSV positive samples. Lanes 11 and 12, negative (no template) controls.

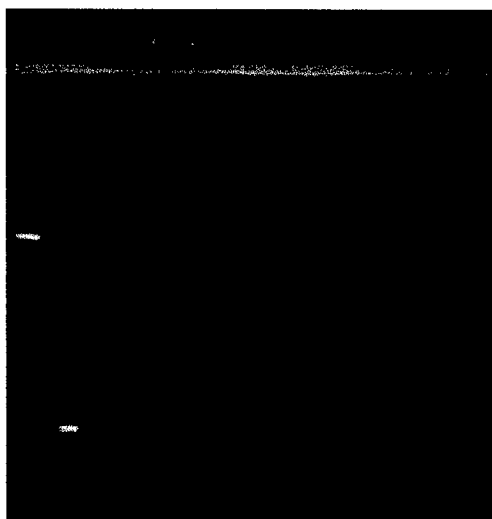


Figure 2. Agarose Gel Electrophoresis of Herpes Virus Polymerase Gene Targets.

Electrophoretic analysis of Herpes simplex type 1 and 2 (235 bp) and Human Herpes type 5 (238 bp) amplicons from formalin-fixed brain tissue samples was carried out as previously described. Lane 1, 100-bp molecular weight ladder. Lane 2, HSV1 (235 bp) positive control. Lane 3, HSV2 (235 bp) positive control. Lane 4, HHV5 (238 bp) positive control. Lanes 5-10, Viral polymerase gene target amplicons from formalin-fixed tissue samples. Lanes 11 and 12, negative (no template) controls.

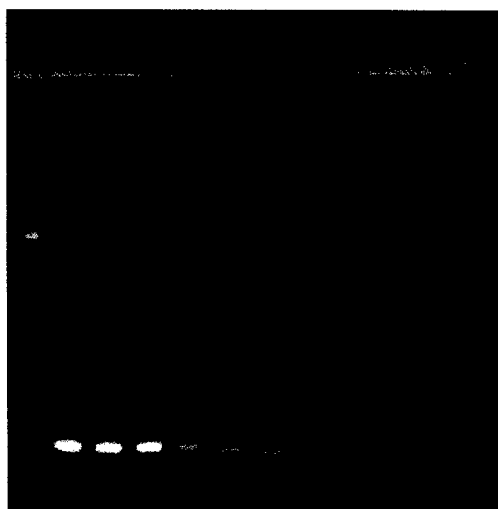


Figure 3. NOS1 Gene Target Sequence. The primers (underlined) give rise to a 251 bp amplicon.

5'GGCCAACAATTCCCTCATCAGCAATGATCGCAGCTGGAAGAGAAACAAGTTCCGC
CTCACCTTTGTGGCCGAAGCTCCAGAACTCACACAAGGTACCTCCCACCACCCTTCC
AGGGAGGTATTCGTGACAATGGGGGAAAGGCAGGTGATCTCACCTCCAGAAGGTGC
TTAGAGAAGATGGGCTTCATTGCTTTGATCTCCCTTTAGTGCAGTCCTACCAAAATG
AATACTTTGCTGCTCTCCCACACTCA3'

Figure 4. Sequence of HSV1, HSV2, and HHV5 Polymerase Gene Target Amplicons. The same set of universal primers was used for all three viruses. The primers (underlined) give rise to a 235 bp amplicon for HSV1 and HSV2, and a 238 bp amplicon for HHV5.

HSV1:

5'TCATCTACGGGGACACGGACTCCATATTTGTGCTGTGCCGCGGCCTCACGGCCGCC
GGGCTGACGGCCATGGGCGACAAGATGGCGAGCCACATCTCGCGCGCGCTGTTTCT
GCCCCCATCAAACCTCGAGTGCAGAAAAGACGTTACCAAACCTGCTGCTGATCGCCAA
GAAAAAGTACATCGGCGTCATCTACGGGGGTAAGATGCTCATCAAGGGCGTGGATC
TGGTGCGCAA3'

HSV2:

5'TCATCTACGGGGACACGGACTCCATTTTCGTTTTGTGCCGCGGCCTCACGGCCGCG
GGCCTGGTGGCCATGGGCGACAAGATGGCGAGCCACATCTCGCGCGCGCTGTTTCT
CCCCCGATCAAGCTCGAGTGCAGAAAAACGTTACCAAAGCTGCTGCTCATCGCCA
AGAAAAAGTACATCGGCGTCATCTGCGGGGGCAAGATGCTCATCAAGGGCGTGGAT
CTGGTGCGCAA3'

HHV5:

5'TCATCTACGGGGACACGGACAGCGTGTTTGTCCGCTTTCGTGGCCTGACGCCGCAG
GCTCTGGTGGCGCGTGGGCCAGCCTGGCGCACTACGTGACGGCCTGTCTTTTGTG
GAGCCCGTCAAGCTGGAGTTTGAAAAGGTCTTCGTCTCTCTTATGATGATCTGCAAG
AAACGTTACATCGGCAAAGTGGAGGGCGCCTCGGGTCTGAGCATGAAGGGCGTGGGA
TCTGGTGCGCAA3'

Sample Number	NOS1 Gene	HSV PCR	Herpes Type
7251	Positive	Positive	HSV1
34302	Positive	Positive	HSV1
81006	Positive	Positive	HSV1
32538	Positive	Positive	HSV2
40075	Positive	Positive	HSV2
42578	Positive	Positive	HSV2
64141	Positive	Positive	HSV2
97104	Positive	Positive	HSV2
13055	Positive	Positive	HHV5
93919	Positive	Positive	HHV5
63537	Positive	Positive	HHV5

Table1: Herpes Virus Positive Sequence Results.

Herpesvirus	Results	Number of Samples	% of Total Samples
HSV1	Positive	3	5.3
	Negative	54	94.7
HSV2	Positive	5	8.8
	Negative	52	91.2
HHV5	Positive	3	5.3
	Negative	54	94.7

Table 2: Percent Samples Testing Positive for Herpes Virus by Type.

DISCUSSION

HSV1 has been suggested as a factor in AD due to its ubiquitous and neurotropic nature, affecting similar sites as those in AD. A number of studies have been performed on frozen brain tissue sections and CSF in order to assess whether the presence of HSV1 in aged brain is associated with AD. Little research has been carried out in determining the presence of viral DNA in formalin-fixed tissue.

It is known that formalin fixation affects significantly in negative fashion, PCR amplification of gene targets in archived tissue. The DNA extraction method employed in this study resulted in the successful extraction of HSV1, HSV2, and HHV5 DNA residing in formalin-fixed, brain tissue from patients with Alzheimer's disease. The herpes virus template was detectable down to a level of 1 pg/ul. However, virus was not detected in all samples tested. It is possible that the 'multiple stage' nature of AD may affect detection of the virus in tissue samples. It is also possible that the virus might be present in very small, localized regions making detection more difficult (17).

Interestingly, NOS1 was detected, i.e. PCR amplified from all formalin-fixed brain tissue samples in this study. Importantly, the size of the NOS1 amplicon (251 bp) is comparable in size to that of the herpes virus (HSV1 and HSV2, 235 bp and HHV5, 238 bp) amplicons. Thus, the NOS1 proved to be a better internal control than that of the commonly used housekeeping gene, GAPDH for DNA extraction from formalin-fixed brain tissue. In contrast to GAPDH, increased time of storage in formalin did not have an effect on the amplification of NOS1. GAPDH template integrity dramatically decreases with increasing time of storage in formalin suggesting that for some yet to be defined reason, GAPDH is a poor control target in formalin-fixed archived brain. One possible explanation is that the primers used for GAPDH amplification may

be targeting a variable region of the GAPDH gene. Furthermore, it is possible that GAPDH exons are more sensitive to formalin than those coding for NOS1.

This study demonstrates the utility of this method of extraction of DNA from formalin-fixed tissue. Furthermore, the NOS1 gene appears to be a superior control candidate target in archived brain. Considering the large quantity of archived brain available, the findings described here could be useful in assessing the association of infectious agents with specific pathology in archived brain tissue.

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